

## The Predominant eIF4G-Specific Cleavage Activity in Poliovirus-Infected HeLa Cells Is Distinct from 2A Protease

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Human enteroviruses and rhinoviruses rapidly and selectively abolish translation from cellular mRNA upon infection of susceptible cells. Expression of the poliovirus 2A protease (PV 2A<sup>pro</sup>) is sufficient to cause host translation shutoff through cleavage of eIF4G (formerly p220, eIF4g) either directly or indirectly through activation of a cellular factor. Evidence exists for both direct and indirect cleavage mechanisms; however, factors presumed to participate in an indirect mechanism have not yet been purified or defined. Here we show that the dominant eIF4G cleavage activity in lysates from infected HeLa cells was separable from PV 2A<sup>pro</sup> by size exclusion chromatography. 2A<sup>pro</sup> separated into two peak fractions which contained activity which cleaved a peptide substrate derived from the poliovirus polyprotein. These peak 2A<sup>pro</sup> fractions did not cleave eIF4G or an eIF4G-derived peptide, as expected, due to the poor efficiency of direct cleavage reactions. Conversely, fractions which contained peak eIF4G cleavage activity and only trace amounts of 2A<sup>pro</sup> efficiently cleaved a peptide substrate derived from the previously mapped eIF4G cleavage site and also cleaved a peptide derived from the poliovirus 1D2A region. The dominant eIF4G cleavage activity was highly purified through four chromatography steps and found to be devoid of all traces of 2A<sup>pro</sup> or its precursors. Quantitation of 2A<sup>pro</sup> from lysates of infected cells showed that during infections in HeLa cells, 2A<sup>pro</sup> does not reach molar excess over eIF4G, as previously shown to be required for direct eIF4G cleavage *in vitro*. Further, infection of HeLa cells in the presence of 2 mM guanidine-HCl, a potent inhibitor of viral RNA replication, suppressed accumulation of 2A<sup>pro</sup> and its precursor 2ABC below detectable levels but was unable to delay the onset of eIF4G proteolysis *in vivo*. The eIF4G cleavage activity was still easily detectable in *in vitro* assays using fractions from guanidine-treated cells. Thus, the data suggest that poliovirus utilizes two catalytic activities to ensure rapid cleavage of eIF4G *in vivo*. Although it was not directly measurable here, 2A<sup>pro</sup> likely does cleave a portion of eIF4G in cells. However, the data suggest that a cellular factor which can be activated by small quantities of 2A<sup>pro</sup> constitutes the bulk of the eIF4G-specific cleavage activity in infected cells and is responsible for the rapid and efficient eIF4G cleavage activity observed *in vivo*. © 1998 Academic Press

### INTRODUCTION

Most members of the picornavirus family inactivate translation of cellular mRNA in infected cells through a mechanism that includes the proteolysis of eukaryotic translation initiation factor 4G (eIF4G, previously referred to as eIF4g or p220) (Devaney *et al.*, 1988; Etchison and Fout, 1985; Etchison *et al.*, 1982). This protein possesses binding sites for eIF3 as well as the m<sup>7</sup>GTP cap-binding protein, eIF4E (Lamphear *et al.*, 1995; Pestova *et al.*, 1996), thus providing a link between capped cellular mRNA and eIF3 complexed with the 40S ribosomal subunit. Within 2 h after enterovirus infection of most susceptible cell types all the eIF4G is rapidly converted into a set of smaller, antigenically related cleavage products. At nearly the same time host protein synthesis is abruptly halted and viral mRNA translation continues via a cap-independent mechanism in which ribosomes bind through recognition of internal viral

RNA sequences (See Belsham and Sonenberg, 1996; Ehrenfeld, 1996, for recent reviews). Interestingly, in some experimental systems host protein synthesis is suppressed by only 50% or less, despite complete eIF4G cleavage (Benton *et al.*, 1995; Bonneau and Sonenberg, 1987; Lloyd and Bovee, 1993; Lloyd *et al.*, 1987; Perez and Carrasco, 1991). This imperfect correlation between eIF4G cleavage and host cell translational shutoff suggests that while eIF4G cleavage is required for shutoff to occur, at least one other event is involved in the total inactivation of translation of host-encoded mRNA in infected cells.

Over the past decade the central role of the viral 2A protease (2A<sup>pro</sup>) in eIF4G cleavage has been investigated, and data acquired using different experimental approaches have led to the proposal of two models for eIF4G cleavage. It has been clearly demonstrated that the poliovirus (PV) 2A<sup>pro</sup> is the only viral protein required for activating eIF4G cleavage (Bernstein *et al.*, 1985; Kräusslich *et al.*, 1987; Lloyd *et al.*, 1988). However, early on it was shown that the majority of PV 2A<sup>pro</sup> does not comigrate with eIF4G-specific cleavage activity upon subcellular fractionation of infected cells, and an anti-2A

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TABLE 1  
Subcellular Fractionation of 2A<sup>pro</sup> and eIF4G Cleavage Activity

Fraction <sup>b</sup>	S10	P10	S200	SW Ribo	RSW
% Total 2A content	46	54	10	32	3.3
eIF4G cleavage activity (U/10 <sup>9</sup> cell eq.)	14.3	4.5	4.7	0.1	9.6
% Total eIF4G cleavage activity	76	24	25	<1	51

*Note.* Values were quantitated using densitometry of scanned immunoblots and autoradiographs with the program NIH Image. Values were averaged from several experiments and are expressed in terms of cleavage units per 10<sup>9</sup> cell equivalents and the percentage of the total found in the whole cell. Activity units were estimated from immunoblots of cleavage reactions containing dilutions of cell fractions and are defined as the amount of cleavage activity required to cleave 50% of the eIF4G present in a 5-ml ribosomal salt wash sample in 90 min at 37°C.

<sup>a</sup> Fractions were derived as under Materials and Methods: S10, postmitochondrial supernate; P10, postmitochondrial pellet; S200, post ribosomal supernate; SW Ribo, salt-washed ribosome pellet, RSW, ribosomal salt wash.

antisera which blocks viral polyprotein processing by 2A<sup>pro</sup> does not inhibit eIF4G cleavage *in vitro* (Lloyd *et al.*, 1986; Kräusslich *et al.*, 1987). Conversely, several recent studies have demonstrated that the purified 2A<sup>pro</sup> of coxsackievirus type B4 (CVB) and human rhinovirus (HRV) (Haghighat *et al.*, 1996; Lamphear *et al.*, 1993; Liebig *et al.*, 1993; Sommergruber *et al.*, 1994), as well as poliovirus (PV) (Bovee *et al.*, 1998), can utilize eIF4G as a substrate *in vitro*, although the cleavage efficiency of these reactions was very low. While these reports showed that direct catalysis of eIF4G cleavage can occur *in vitro*, the inefficiency of reactions appear inconsistent with the rapid and efficient cleavage of eIF4G observed *in vivo* for these picornaviruses (Etchison *et al.*, 1982; Kirchweiger *et al.*, 1994). Thus, it is not known whether direct cleavage by 2A<sup>pro</sup> simply occurs much more efficiently *in vivo* than has been measured *in vitro* reactions due to alteration of eIF4G substrate or a unique micro-environment or whether a more efficient cleavage mechanism involving a cellular factor is operative *in vivo*. Furthermore, since the data supporting the involvement of an additional cellular factor (an indirect cleavage mechanism) have been derived solely from studies of PV-infected cells, it has been proposed (Haller and Semler, 1995) that PV uses a mechanism for activation of eIF4G proteolysis different from that employed by CVB and HRV.

For this study, we examined in more detail the eIF4G-specific cleavage mechanism in PV-infected HeLa cells. In particular, we focused on the relative contribution of 2A<sup>pro</sup> and other proteolytic factors to the total eIF4G cleavage observed. We report here that radiolabeled PV 2A<sup>pro</sup> did not copurify through size exclusion chromatography with the eIF4G-specific cleavage activity measurable in infected HeLa cell lysate. We also purified the dominant eIF4G-specific cleavage activity from PV-infected cells to a very high level and showed that it contained no measurable 2A<sup>pro</sup>. In addition we determined that the level of 2A<sup>pro</sup> present in PV-infected HeLa cell lysate at early or late timepoints during infection was insufficient to cleave significant amounts of eIF4G via

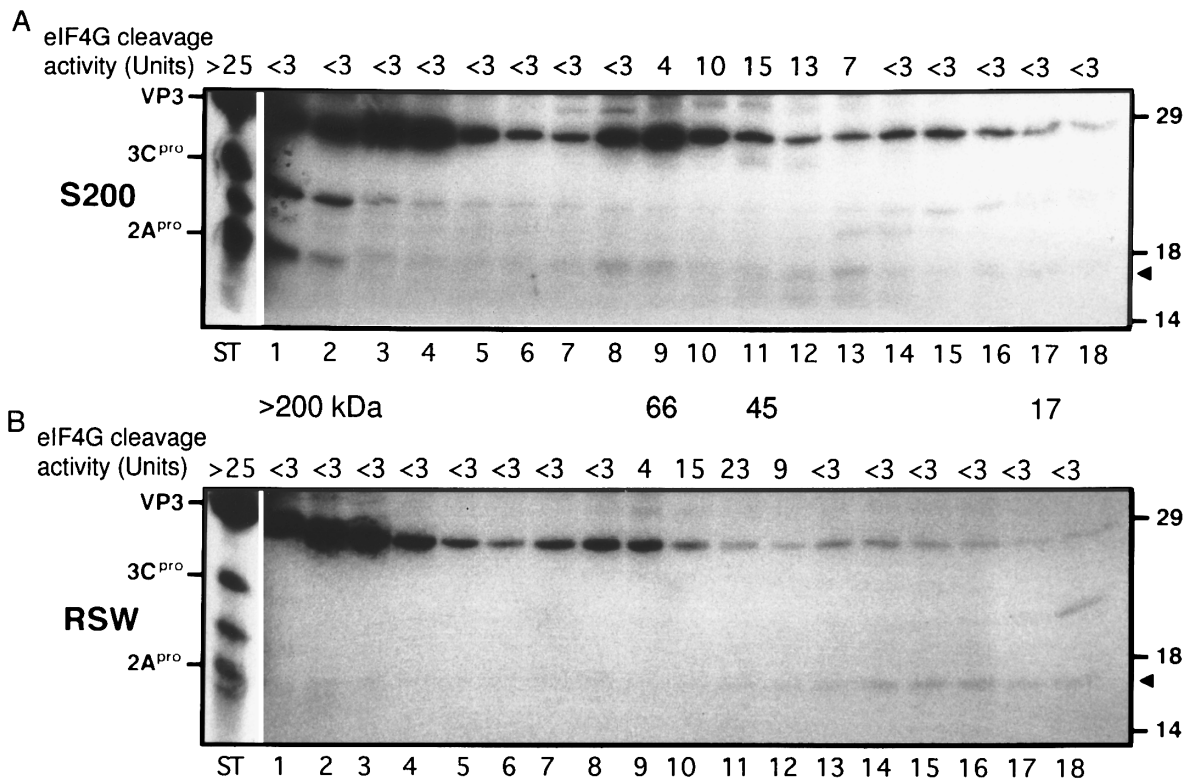
direct catalysis. Last, we showed that the presence of guanidine during infection drastically reduced production of 2A<sup>pro</sup> to undetectable levels, yet did not significantly delay the rapid *in vivo* cleavage of eIF4G. These data strongly suggest that a highly active cellular protease supplements 2A<sup>pro</sup> to result in the efficient *in vivo* cleavage of eIF4G.

## RESULTS

### The primary eIF4G cleavage activity in infected cell lysate segregates from PV 2A<sup>pro</sup>

Purified and/or recombinant forms of 2A<sup>pro</sup> and eIF4G have been used previously to demonstrate that direct proteolysis of eIF4G occurs *in vitro* (Haghighat *et al.*, 1996; Lamphear *et al.*, 1993; Liebig *et al.*, 1993; Sommergruber *et al.*, 1994); however, typical *in vitro* cleavage reactions required high enzyme concentrations and molar excess of 2A<sup>pro</sup> over substrate to cause complete cleavage. This is in contrast to the rapid and efficient eIF4G cleavage observed *in vivo* (Etchison *et al.*, 1982; Kirchweiger *et al.*, 1994).

To address the issue of *in vivo* eIF4G cleavage, we have focused on the dominant eIF4G cleavage activity which can be easily detected in infected cells and tested whether this activity was associated with 2A<sup>pro</sup>. We also wanted to test whether native 2A<sup>pro</sup> in cell lysates could be demonstrated to contain this dominant cleavage activity. Thus, we have more completely characterized the subcellular fractionation of PV 2A<sup>pro</sup>, which was previously shown to be evenly distributed between the insoluble membrane-containing P10 compartment and the soluble S10 compartment. Further fractionation of the S10 into the high-speed supernate (S200), ribosomal salt wash (RSW), and washed ribosome pellet (SWRibo) fractions allowed enrichment of eIF4G cleavage activity which could be further concentrated by 40% ammonium sulfate precipitation (Lloyd *et al.*, 1987). These fractions were also assessed here for both eIF4G cleavage activity and presence of 2A<sup>pro</sup>, as shown in Table 1. The data show that while 2A<sup>pro</sup> associates closely with insoluble



**FIG. 1.** Size exclusion chromatography of radiolabeled PV-infected HeLa lysate fractions. Radiolabeled S200 A-cut (top) and the RSW A-cut (bottom) subcellular fractions were separately chromatographed on an SW3000 column and fractions were subjected to SDS-PAGE and autoradiographed for 25 days, as described under Materials and Methods. Short exposures of the respective HPLC start samples are shown (lane ST). 35 ml of each fraction was assayed for eIF4G cleavage activity as described in Table 1 and total units were calculated for the entire fraction. Chromatographic migration peaks for molecular weight standards are indicated between panels: blue dextran, >200 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; RNase A, 17 kDa. SDS-PAGE mobilities of 2A<sup>pro</sup> and other viral proteins are labeled relative to molecular weight standards. 2A<sup>pro</sup> is marked by an arrowhead.

proteins and membranes in the P10 and SWRibo pellet fractions, the majority of eIF4G cleavage activity is associated with the soluble S200 and RSW fractions. In particular the SW Ribo fraction contains approximately 32% of the total 2A<sup>pro</sup> content of the cell, yet no eIF4G cleavage activity is detected in this fraction. No fractions prepared in a similar fashion from mock-infected cells contain detectable eIF4G cleavage activity.

We then tested whether eIF4G cleavage activity could be chromatographically separated from 2A<sup>pro</sup>. The S200 A-cut and RSW A-cut fractions were chosen because they contained the most concentrated eIF4G cleavage activity and limited but detectable amounts of 2A<sup>pro</sup>, and their purity relative to crude lysate allowed better resolution by HPLC. These samples were independently chromatographed on a SW3000 gel filtration column (Fig. 1). Figure 1A shows the elution profile of radiolabeled 2A<sup>pro</sup> from the S200 A-cut fraction. Interestingly, 2A<sup>pro</sup> in the S200 fraction was detected in all fractions, yet concentrated into several peaks which eluted with apparent molecular masses of approximately 200, 75, 30, and 17 kDa, from left to right (lanes 1–2, 8–9, 13, 16–17). The majority of 2A<sup>pro</sup> was found in the large molecular weight fraction (lanes 1–2), apparently in some state of aggre-

gation or complexed with other unknown molecules. Importantly, neither this fraction, nor any of the other peak fractions enriched for 2A<sup>pro</sup>, possessed measurable eIF4G cleavage activity. Unlike the polyphasic migration of 2A<sup>pro</sup>, eIF4G cleavage activity was centered around a single peak near 50 kDa (lanes 10–12) and did not correspond to fractions containing the most 2A<sup>pro</sup>. Similar analysis of the chromatographed RSW A-cut (Fig. 1B) showed 2A<sup>pro</sup> migrating almost exclusively in the expected 17-kDa range, but the eIF4G cleavage activity peak again was associated with fractions in the 50-kDa size range, which contained only trace levels of 2A<sup>pro</sup>. Although some direct cleavage of eIF4G by the 2A<sup>pro</sup>-enriched column fractions might have been expected, it was likely not observed because we and others have shown previously that substantial direct catalysis of eIF4G cleavage requires molar excess of 2A<sup>pro</sup>, which was not present in these assays (Bovee *et al.*, 1998; Haghighat *et al.*, 1996; Lamphear *et al.*, 1993; Liebig *et al.*, 1993; Sommergruber *et al.*, 1994). Importantly, analysis of autoradiographs also revealed that the 2A<sup>pro</sup> precursor 2ABC was only detected in high-molecular-weight fractions (S200 fractions 1–9 or RSW fractions 1–5); thus,

TABLE 2

## Protease Cleavage Assays Using Pooled HeLa Cell Fractions

Substrate	Fractions <sup>a</sup>		
	2A <sup>pro</sup> (HMW) (70%)	eIF4G cleavage (3.8%)	2A <sup>pro</sup> (LMW) (26%)
1D2A peptide	26%	84%	27%
H-4G peptide	9.3%	91%	11%
eIF4G	0%	97%	1%

Note. Cleavage assays are expressed as percentage cleavage of input substrate peptide or eIF4G protein.

<sup>a</sup> Pooled fractions include apparent high-molecular-weight (HMW) and low-molecular-weight (LMW) 2A<sup>pro</sup> fractions and the fraction containing peak eIF4G cleavage activity. The percentage of total 2A<sup>pro</sup> from the starting S10 lysate that was present in each fractionated pool is listed and was estimated from autoradiographs using NIH Image densitometry software.

2ABC did not comigrate with eIF4G cleavage activity (data not shown).

### 2A<sup>pro</sup>-enriched cell fractions cleave a viral polyprotein-derived peptide but not an eIF4G-derived peptide

We have previously used a peptide containing the native 1D2A junction polyprotein cleavage site sequence for sensitive assay of 2A<sup>pro</sup> activity (Alvey *et al.*, 1991; Bovee *et al.*, 1998). Further we have recently shown that this assay is at least a 10-fold more sensitive indicator of 2A<sup>pro</sup> activity than eIF4G cleavage assays, requiring only 1 nM enzyme (Bovee *et al.*, 1998). Thus, we attempted to probe for trace amounts of highly active 2A<sup>pro</sup> in fractions containing peak eIF4G cleavage activity by assaying for proteolysis of the PV 1D2A peptide. For these activity assays the crude PV-infected S10 lysate was passed over a Sephacryl S300 size exclusion column. The elution profile was similar to those in Fig. 1, with 70% of 2A<sup>pro</sup> eluting with the highest molecular weight complexes (HMW pool) and a smaller proportion (26%) having the same elution time as a 17-kDa protein standard (LMW pool). eIF4G cleavage activity eluted just before the 17-kDa 2A<sup>pro</sup> peak (eIF4G cleavage pool) and contained only a trace of 2A<sup>pro</sup> detectable by autoradiography. Fractions containing peak levels of 2A<sup>pro</sup> or eIF4G cleavage activity were separately pooled and compared in their ability to cleave synthesized peptide substrates derived from either the PV polyprotein or human eIF4G (Table 2). As expected, the H-4G peptide was cleaved significantly only when incubated with the eIF4G cleavage pool, thus reflecting the cleavage pattern observed with eIF4G (RSW) cleavage assays. Again, significant direct cleavage of the 4G peptide was not expected by 2A<sup>pro</sup>-enriched fractions (HMW pool and LMW pool) because molar excess of 2A<sup>pro</sup> did not exist in these as-

says. When the 2A<sup>pro</sup>-enriched fractions were tested with the 1D2A substrate, significant, though incomplete, cleavage was consistently observed in HMW and LMW pools, demonstrating that active 2A<sup>pro</sup> was present in these fractions as expected. Further, the 2A<sup>pro</sup> in HMW and LMW pools exhibits the same preference for proteolysis of the 1D2A peptide over the H-4G peptide (e.g., 26% versus 9% cleavage), as we previously showed using purified recombinant 2A<sup>pro</sup> (Bovee *et al.*, 1998).

Interestingly, the fraction with peak levels of 4G-cleavage activity also exhibited high cleavage activity on the 1D2A substrate. These two peptides share 80% sequence homology over the P4-P1' amino acid residues that provide the bulk of the substrate recognition and specificity information for most cysteine proteases. Therefore, just as 2A<sup>pro</sup> is capable of cleaving the alternate eIF4G sequence, a cellular eIF4G-specific protease may be predicted to be capable of cleavage of a related 1D2A peptide with some efficiency. However, it is also possible that the HMW and LMW fractions may contain an inhibitor of 2A<sup>pro</sup> with partial activity. The data in this figure cannot distinguish between these two possibilities. However, the demonstration of significant 2A<sup>pro</sup> activity in both HMW and LMW fractions also indicates that if a protease inhibitor is present, it is insufficient to effectively block 2A<sup>pro</sup> activity. Also, a putative partial inhibitor cannot readily explain the lack of eIF4G cleavage in those same fractions. Further, the results also confirm that H-4G peptide cleavage activity comigrates only with fractions shown to catalyze eIF4G cleavage. Taken together, the simplest interpretation of the data is that two cleavage activities exist and that a protease or factor with high activity which does not comigrate with 2A<sup>pro</sup> is responsible for the majority of eIF4G- and H-4G-specific proteolytic activity measurable in lysates from infected cells.

### Purification of eIF4G cleavage activity correlates with loss of detectable 2A<sup>pro</sup>

Based on the above observations, efforts were focused on further purification of the eIF4G cleavage activity. Table 3 lists the sequential steps developed for a larger scale purification scheme. Viral proteins were radiolabeled from 3.5 to 5 hpi before preparation of extracts for the purification process and active fractions from each purification step were pooled and examined by autoradiography for 2A<sup>pro</sup> or precursors such as 2ABC. Figure 2 shows analysis of the same purified pools listed in Table 3 and shows that after the second fractionation step, on Fast Q Sepharose, no radiolabeled 2A<sup>pro</sup> or unprocessed 2ABC precursor protein was detectable in the pool that exhibited peak eIF4G cleavage activity. Further purification over nickel and heparin columns reduced the number of HeLa cell proteins detect-

TABLE 3

## Purification of eIF4G Cleavage Activity

Procedure	Total protein (mg)	Purification <sup>a</sup> (-Fold)	Specific activity <sup>b</sup> U/mg
RSW-A cut	33	50	2.6
Sephacryl S300	9.4	175	56
Fast Q Sepharose	0.82	2,012	153
Nickel affinity	0.16	10,062	1687
Heparin-Sepharose	0.032	50,312	6250

<sup>a</sup> 0–40% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitate of ribosomal salt wash typically averages 50-fold purification from crude HeLa cell lysate (by protein stain (Pierce)).

<sup>b</sup> Activity estimated from immunoblots, defined as the amount of cleavage activity required to cleave 50% of the eIF4G present in a ribosomal salt wash sample in 90 min at 37°C.

able by silver stain to less than 20 (data not shown). This further fractionation also removed all detectable viral proteins except for a trace of 3CD which was revealed only on a 19-day autoradiograph exposure (too faint to reproduce well in Fig. 2). Although the precise quantitation of cleavage activity was not possible from these assays, the recovery of activity remained high after each additional fractionation step. These data show that the primary eIF4G cleavage activity in infected cell lysates can be separated from all detectable traces of 2A<sup>pro</sup> and comigrates with a small subset of HeLa cell proteins.

#### Quantitation of 2A<sup>pro</sup> from infected cell lysate indicates an insufficient level for direct proteolysis of eIF4G

In a previous report, we demonstrated that while *in vitro* proteolysis of eIF4G can be directly catalyzed by recombinant PV or CVB 2A<sup>pro</sup>, the reaction requires high concentrations and molar excess of enzyme (Bovee *et al.*, 1998). If efficiency of the direct cleavage reaction observed *in vitro* reflects direct eIF4G cleavage *in vivo* (which is not known at this time) then similarly high molar ratios of 2A<sup>pro</sup> to eIF4G should exist in lysates from infected cells. Production of 2A<sup>pro</sup> is not detectable by autoradiography of proteins from pulse-labeled cells until after host protein shutoff occurs (near 2.5 hpi); then the amount of 2A<sup>pro</sup> accumulates until 4.5 hpi (see Fig. 4). Despite several attempts, 2A<sup>pro</sup> was undetectable by conventional Western blot (which will detect as little as 80 ng 2A<sup>pro</sup>) at any timepoint during the infection (data not shown). Therefore, a light-generating immunodetection system was used in 2A<sup>pro</sup> immunoblots (Super Signal Ultra, Pierce) which extended the assay sensitivity over 50-fold. The 2A<sup>pro</sup> signal in S10 or P10 lanes was calibrated against a dilution series of a known concentration of recombinant 2A<sup>pro</sup> previously quantitated by Coomassie stain relative to known standards (Bovee *et al.*, 1998). By comparison with purified eIF4G standards,

we have previously determined that 1 ml of S10 from uninfected HeLa cells contains approximately 36 ng of eIF4G, or about 0.23 pmol (Bovee *et al.*, 1998). Figure 3 shows a titration of the soluble (S10) and insoluble (P10) contents of PV-infected HeLa cells at 5 hpi, when the highest levels of 2A<sup>pro</sup> have accumulated. The data show that the total 2A<sup>pro</sup> synthesized *in vivo* was nearly equivalently distributed between these compartments (as shown in Table 1) and corresponded to a maximum of about 1 ng/ml (0.06 pmol/ml) in each compartment (compare lane 4 to lanes 9 and 12). In addition to reactivity to 2A<sup>pro</sup>, the polyclonal serum contains reactivity against a bacterial protein which migrates near 20 kDa, and under the Super Signal Ultra immunoblot conditions, a different

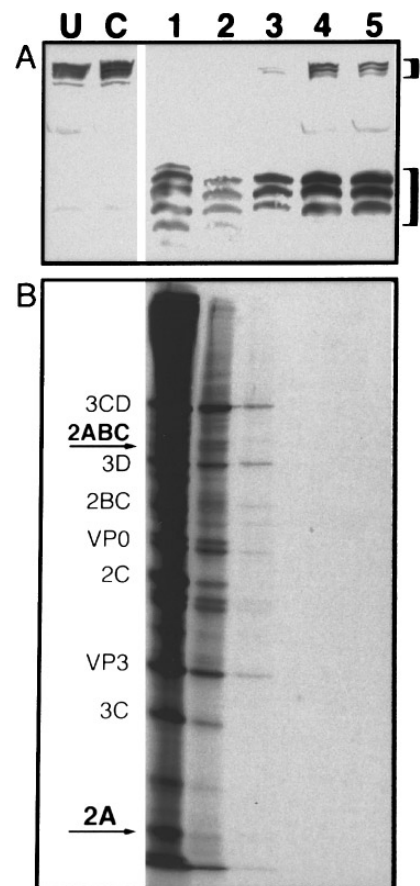


FIG. 2. Purification of eIF4G cleavage activity from radiolabeled PV-infected HeLa cells. (A) eIF4G cleavage activity assays (lanes 2–5) on material pooled at each purification step. Lanes contain: U, unincubated RSW substrate control; C, RSW substrate incubated 2 h at 37°C in the absence of material from infected cells; 1, 7 ml RSW from PV-infected cells (eIF4G cleavage product marker); 2, Sephacryl S300 pool; 3, Fast Q Sepharose pool; 4, nickel-affinity pool; and 5, heparin-Sepharose pool. Lanes 1–5 contain approximately 2.3, 0.5, 1.5, 1.5, and 3% of each respective total protein sample. Samples were subjected to 7% SDS-PAGE and transferred to nitrocellulose, and eIF4G (short bracket) or its cleavage products (long bracket) were visualized by immunoblot. (B) 4-day autoradiograph showing PV proteins detectable in the corresponding pooled samples from A, lanes 2–5. The major PV proteins are labeled.

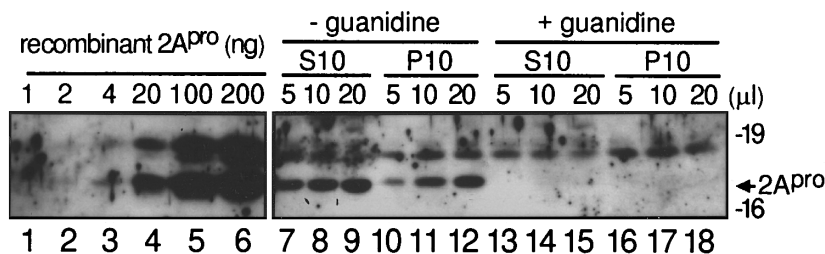


FIG. 3. Quantitation of 2A<sup>pro</sup> from PV-infected HeLa lysate at 5 hpi. Cells infected in the absence or presence of 2 mM guanidine were lysed and processed into soluble (S10) and insoluble (P10) compartments. Each sample was titrated on 12% SDS-PAGE along with recombinant 2A<sup>pro</sup> of known concentration (see Materials and Methods) and visualized on X-ray film using a light-generating immunoblot procedure. Upper bands migrating at 20 kDa in *Escherichia coli* preparations (lanes 1–6) or at 19 kDa in HeLa lysate (lanes 7–18) are unrelated cellular proteins that react with IgG in the same serum. Positions of 2A<sup>pro</sup> and molecular weight standards are indicated.

reactivity against an unknown HeLa protein also emerged (Fig. 3, upper bands). Thus, the data from this experiment reflected a molar enzyme to substrate ratio in the cell at 5 h p.i. of approximately 1:4, which is approximately 20-fold lower than ratios which were found to be required to catalyze complete direct cleavage of eIF4G *in vitro* with purified recombinant PV, CVB4, or HRV2 2A<sup>pro</sup> produced in several laboratories (Bovee *et al.*, 1998; Haghighat *et al.*, 1996). The levels of 2A<sup>pro</sup> in cells during the early period of 1.5–2.5 h pi when eIF4G is actually

cleaved are unknown, but will be significantly lower than 1 ng/ml (see Fig. 4).

#### Reduction of 2A<sup>pro</sup> synthesis in infected cells does not delay eIF4G proteolysis

The molar enzyme to substrate ratio in infected cells can be altered by infecting HeLa cells with PV in the absence or presence of 2 mM guanidine, which drastically inhibits viral RNA replication through its effect on

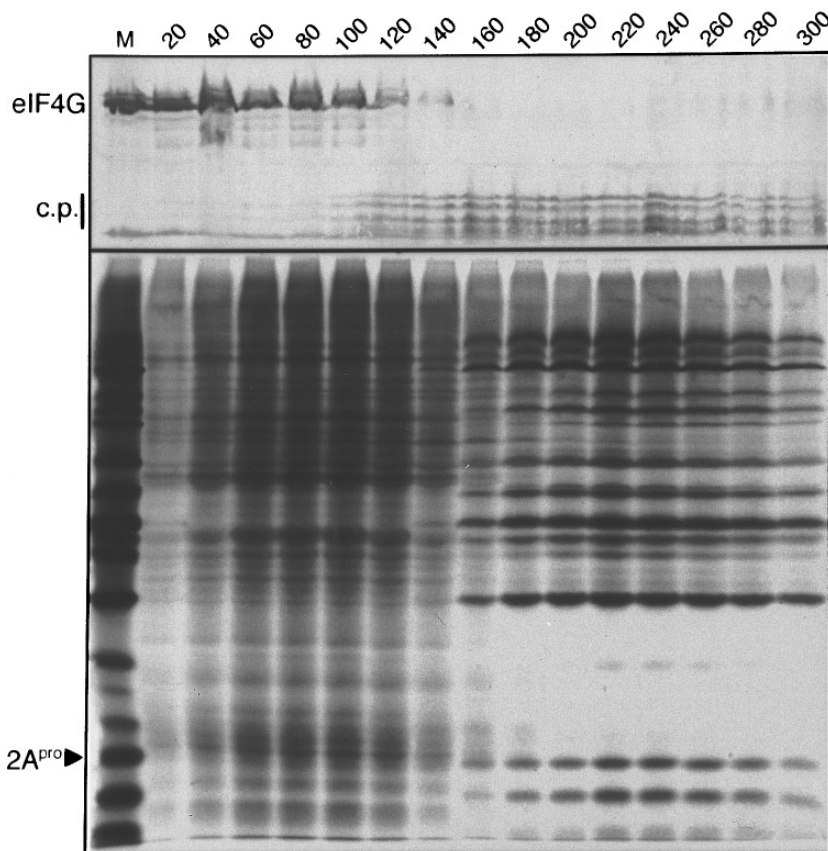


FIG. 4. eIF4G cleavage kinetics and host cell shutoff in PV-infected HeLa cells. (Top) Western blot with eIF4G antiserum shows distribution of intact (4G) and cleaved (c.p.) eIF4G at 20 min intervals from 0 to 5 hpi. (Lane M) eIF4G from mock-infected cells. (Bottom) Autoradiogram from a pulse label experiment shows proteins synthesized during 20-min intervals from 0 to 5 hpi. (Lane M) Position of 2A<sup>pro</sup> is indicated in the PV protein marker.

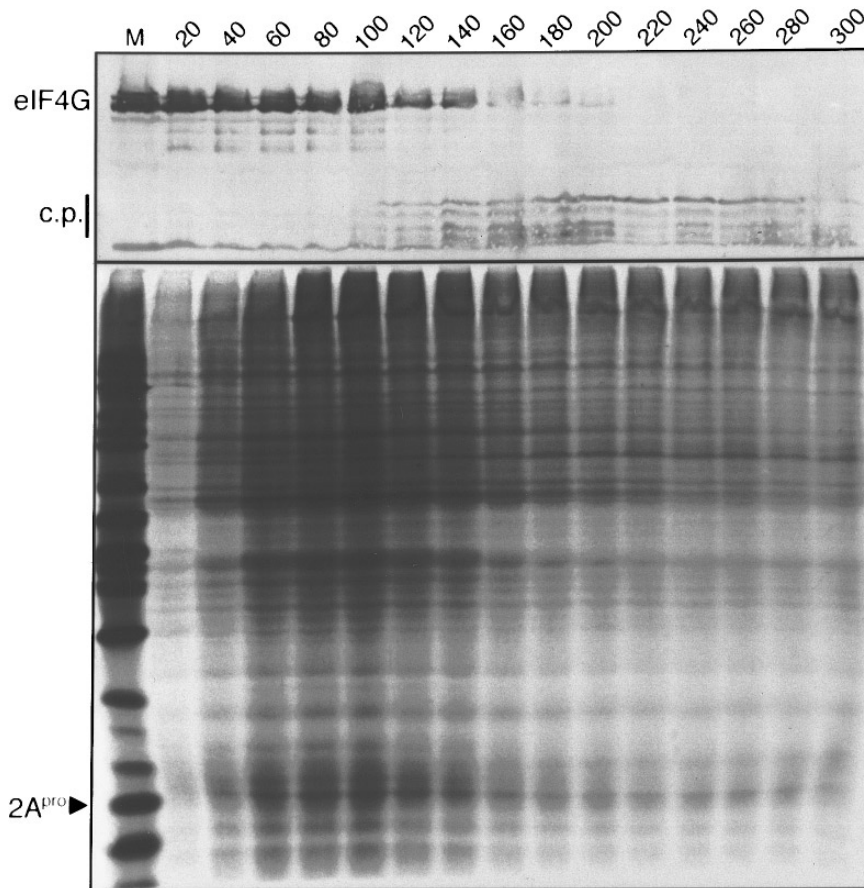


FIG. 5. eIF4G cleavage kinetics and host cell shutoff during infection in the presence of guanidine. Same as legend to Fig. 4, except the virus infection contained 2 mM guanidine, as described under Materials and Methods.

the function of viral protein 2C (Baltera and Tereshak, 1992; Tolskaya *et al.*, 1994). By eliminating the amplification of viral RNA, guanidine also greatly reduces the production of 2A<sup>pro</sup> and all other viral proteins in infected cells since only the input virion mRNA can be translated. Samples from infected cells were monitored at 20-min intervals to examine the kinetics of 2A<sup>pro</sup> expression and the extent of eIF4G proteolysis, as described under Materials and Methods. Figure 4 shows that in the absence of guanidine, synthesis of 2A<sup>pro</sup> first became detectable

at 160 min pi, concurrent with the shutoff of cellular protein synthesis. eIF4G cleavage was first observed at 100 min pi, 60 min before 2A<sup>pro</sup> was detected. Figure 5 shows that addition of guanidine to infected cells reduced production of all viral proteins to levels below detection by autoradiography, as expected. We wished to determine whether 2A<sup>pro</sup> could be detected before 160 min in control infections, or at any timepoint for cells infected in the presence of guanidine, in case significant levels were present but simply masked by the translation

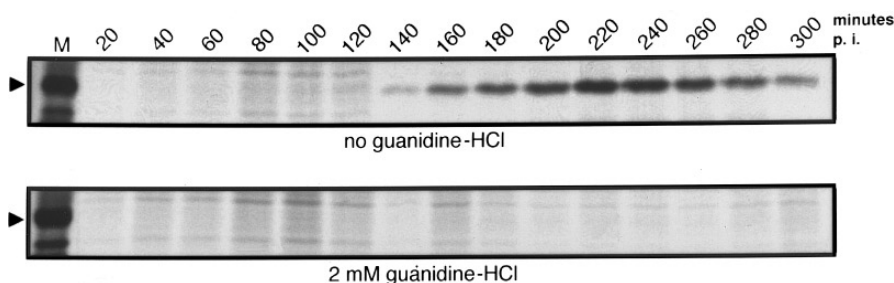


FIG. 6. Completion of eIF4G cleavage coincides with earliest detection of 2A<sup>pro</sup> by immunoprecipitation. All pulse label samples shown in Figs. 4 and 5 were subjected to immunoprecipitation as described under Materials and Methods and 2A<sup>pro</sup> was visualized by 12% SDS-PAGE and autoradiography. Autoradiographs were exposed for 5 weeks, resulting in detection of two background HeLa proteins.



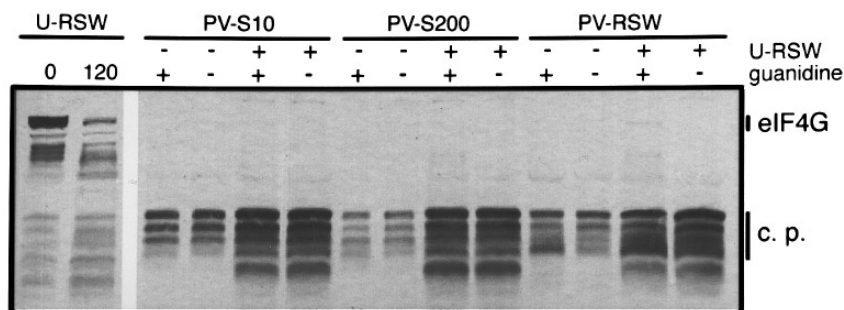


FIG. 7. Efficient *in vitro* cleavage of eIF4G in the absence of detectable levels of PV 2A<sup>pro</sup>. Infected cells were processed as previously described and then combined with exogenous intact eIF4G present in the RSW fraction from uninfected HeLa cells (U-RSW). Cleavage assays were stopped after 120 min at 37°C by boiling in denaturing gel loading buffer. Samples were then subjected to SDS-PAGE and immunoblotted as described above. U-RSW before (0) and after (120) incubation.

of cellular proteins. To test this, radioimmunoprecipitation (RIP) was performed on pulse-labeled samples from the infections shown in Figs. 4 and 5. Figure 6 shows that RIP extended the earliest detection of 2A<sup>pro</sup> in a normal infection to 120 min pi, 40 min earlier than on autoradiographs alone (compare with Fig. 4); however, 2A<sup>pro</sup> was undetectable in guanidine-treated cells by this method at any timepoint, even upon extended exposures (Fig. 6). Figure 3 also shows that accumulation of 2A<sup>pro</sup> was undetectable by immunoblot methodology at 5 hpi in lysates from guanidine-treated cells (lanes 13–18). The precise amounts of 2A<sup>pro</sup> produced in these cells is not known but is at least 20-fold reduced by guanidine treatment (Fig. 3).

Importantly, addition of guanidine caused only a slight change in the kinetics of eIF4G cleavage. With or without guanidine, the onset of eIF4G cleavage was first detectable at the same time, 100 min pi, although complete cleavage in guanidine-treated cells was delayed slightly. In either case, eIF4G cleavage began before production of 2A<sup>pro</sup> was detectable. Therefore, rapid, efficient eIF4G cleavage occurs *in vivo* under conditions where 2A<sup>pro</sup> is completely undetectable. Also, as previously shown by others, complete proteolysis of all detectable eIF4G did not result in complete host cell translation shutoff, suggesting that complete shutoff of cellular translation requires at least one event in addition to eIF4G cleavage (Bonneau and Sonenberg, 1987; Lloyd *et al.*, 1987; Pérez and Carrasco, 1992).

#### *In vitro* eIF4G cleavage activity in cellular lysates is not diminished by reduction in levels of 2A<sup>pro</sup>

We also examined the effect of altering the molar ratio of 2A<sup>pro</sup> and eIF4G in cell lysates on the *in vitro* eIF4G cleavage activity. Thus, the predominant eIF4G cleavage activity was measured in lysates from control or guanidine-treated infected cells. Figure 7 shows that guanidine treatment caused no discernable reduction in the level of *in vitro* eIF4G cleavage activity detected in any of the three subcellular compartments which were as-

sayed, despite the corresponding reduction in 2A<sup>pro</sup>. Taken together, these data support the conclusion that the efficient *in vitro* cleavage of eIF4G measured *in vivo* and *in vitro* does not arise from direct catalysis by 2A<sup>pro</sup> and likely involves a cellular factor.

## DISCUSSION

It is now generally accepted that 2A<sup>pro</sup> is the only viral protein required for cellular translation shutoff to occur and that the hallmark of this phenomenon is the proteolysis of eIF4G. It was originally proposed based on subcellular fractionation experiments and antibody inhibition studies that eIF4G was cleaved in PV-infected HeLa cells via an indirect mechanism involving activation of a cellular factor (Kräusslich *et al.*, 1987; Lloyd *et al.*, 1986). More recently investigations by independent researchers using highly purified eIF4G and CVB4 and HRV2 proteases have clearly shown that eIF4G can be cleaved *in vitro* by CVB or HRV 2A<sup>pro</sup> alone, at a region bearing conserved amino acids important for 2A<sup>pro</sup> substrate recognition on the viral polyprotein (Lamphear *et al.*, 1993; Sommergruber *et al.*, 1994). Based on this observation it was proposed that eIF4G cleavage was catalyzed directly by 2A<sup>pro</sup> in infected cells. This direct mechanism for eIF4G cleavage is very appealing in its simplicity, but conflicts with the earlier observations in which subcellular fractionation of HeLa cell lysates separated eIF4G cleavage activity from the majority of 2A<sup>pro</sup> (Lloyd *et al.*, 1986).

In an effort to determine if PV used a different eIF4G cleavage mechanism than that used by CVB and HRV, we have recently performed new studies which showed that purified, active PV and CVB 2A<sup>pro</sup> both are capable of direct cleavage of purified eIF4G; however, each reaction was inefficient, requiring high enzyme concentrations and a molar excess of enzyme to catalyze complete cleavage (Bovee *et al.*, 1998). This result mechanistically unified the PV and CVB experimental models but also raised questions regarding the physiological significance of the direct cleavage mechanism since complete cleavage of eIF4G in-



fects cells is very rapid and occurs before 2A<sup>pro</sup> is readily detectable. It is possible that *in vitro* measurements of eIF4G cleavage activity catalyzed by 2A<sup>pro</sup> do not accurately reflect the same reaction *in vivo* or that there is a more efficient mechanism involving some other cellular factor operating *in vivo*.

In this report, which focused on the eIF4G cleavage activity measurable in infected HeLa lysates, we showed that PV-infected cells contain an activity which causes eIF4G cleavage, but that this cleavage activity segregates from the majority of active 2A<sup>pro</sup> by several types of chromatography. Further, the eIF4G cleavage activity can be highly purified into fractions containing several host proteins but no trace of 2A<sup>pro</sup> or its precursor 2ABC. We also showed that the 2A<sup>pro</sup> in HeLa cell lysates was catalytically active against a peptide containing the 2A cleavage sequence from the PV polyprotein but does not accumulate to levels high enough to cause measurable eIF4G cleavage in *in vitro* assays. Finally, the inclusion of guanidine to severely limit production of 2A<sup>pro</sup> during infection caused no significant delay in the rapid and specific cleavage of eIF4G *in vivo*. Taken together, these data are not easily reconcilable with a predominantly direct mechanism of eIF4G cleavage *in vivo* and instead strongly suggest the involvement of at least one cellular factor, which is likely to be a protease. We have completed similar experiments with CVB4-infected HeLa cells which also resulted in separation of 2A<sup>pro</sup> from the dominant eIF4G-specific cleavage activity (unpublished observations). It therefore seems likely that all species of enteroviruses utilize a common indirect mechanism for the rapid, complete, *in vivo* proteolysis of eIF4G.

It is likely that eIF4G cleavage *in vivo* is catalyzed via both direct and indirect mechanisms simultaneously; however, for a direct cleavage mechanism to be responsible for the dominant cleavage activity measured in HeLa lysates, an unknown form of 2A<sup>pro</sup> with much higher specific activity for eIF4G must exist in cells, or an alternate form of substrate which has not yet been described or tested is required to enhance direct cleavage reactions (i.e., not purified eIF4G alone or eIF4G found in eIF4F or eIF4F/eIF3 complexes) (Bovee *et al.*, 1998; Haghihat *et al.*, 1996; Lamphear *et al.*, 1993). Some investigators have proposed that a cellular protein may interact with 2A<sup>pro</sup> to massively increase its cleavage activity on eIF4G by either binding 2A<sup>pro</sup> to alter cleavage activity or imparting a covalent modification such as a cleavage event on 2A<sup>pro</sup>. Although this remains a formal possibility, some radiolabeled 2A<sup>pro</sup> should copurify and be concentrated and enhanced over the course of an extensive purification of eIF4G cleavage activity; this has not been seen despite numerous attempts using a large variety of purification schemes.

The data here suggest that indirect cleavage involves a much more efficient catalytic reaction and this activity predominates over the native 2A<sup>pro</sup> direct cleavage re-

action in infected cells. Indeed, our efforts to identify the cellular proteolytic activity in these fractions has proven extremely difficult because of the apparent high specific activity of this enzyme. A two-step or multiple step eIF4G cleavage reaction is very appealing since it greatly increases the efficiency of eIF4G cleavage beyond the capabilities of 2A<sup>pro</sup> alone. Further, there is ample precedence for proteolytic activation cascades throughout biology in the mechanisms of fibrin deposition, complement activation, activation of digestive proteases, and more recently, activation of intracellular caspases during apoptosis (Faleiro *et al.*, 1997).

It is possible that enteroviruses have gained the capability of activating a cellular protease(s) which is normally involved in the regulation of translation or some other process in the uninfected cell. Indeed, the generation of similar eIF4G cleavage products during purification of initiation factors from uninfected cells has been a common unreported problem (data not shown). Further, there have been previous reports of cellular proteases which cleave eIF4G in uninfected cells, including calpain and an activity induced in hemin-differentiated K562 cells (Benton *et al.*, 1996; Wyckoff *et al.*, 1990). In this regard, we have recently discovered that eIF4G is targeted for proteolytic cleavage during the execution phase of apoptosis in uninfected cells and that at least two distinct cellular eIF4G-specific proteases are activated (submitted for publication). One of these activities produces cleavage products which comigrate with eIF4Gcp from poliovirus-infected cells (submitted for publication). We are pursuing the possibility that the predominant eIF4G cleavage activity described here may be a caspase activated by poliovirus infection instead of apoptosis.

It is interesting to speculate how two modes of eIF4G cleavage evolved. Recent studies have shown that eIF4G must be in a form complexed with eIF4E to serve as a substrate for 2A<sup>pro</sup> (Haghighat *et al.*, 1996; Ohlmann *et al.*, 1997). In contrast, the apparent cellular protease activated by 2A<sup>pro</sup> efficiently cleaves all the eIF4G present in lysates, in all its possible forms. Finally, we have recently shown in a K562 persistent infection model that complete cleavage of eIF4G and development of host translation shutoff are not required for viral RNA replication and growth. In contrast, the ability to cause complete shutoff was closely linked to cell lysis (Benton *et al.*, 1995; Benton *et al.*, 1996; Lloyd and Bovee, 1993). Thus it is possible that enteroviruses acquired the ability to cause complete cleavage of eIF4G and host translation shutoff in an effort to aid lysis and viral spread and/or to quickly block development of interferon responses in the alimentary tract. Thus, the phenomenon of host translation shutoff may exist primarily for viral pathogenesis.

## MATERIALS AND METHODS

### Cells and virus

HeLa S3 tissue culture cells originally obtained from the American Type Culture Collection were grown at 37°C in Joklik's SMEM supplemented with 9% bovine calf and 1% fetal bovine serum at a density ranging from  $2 \times 10^5$  to  $8 \times 10^5$  cells per milliliter. Poliovirus type 1 (Mahoney strain) was cultivated in HeLa cells and purified as previously described (Jones and Ehrenfeld, 1983). Depending on the experiment, infections were carried out at multiplicities of infection of 30- or 100 PFU/cell in a 37°C recirculating water bath.

### Radiolabeling of viral proteins

Viral proteins were radiolabeled *in vivo* by incubating infected HeLa cells in methionine-free SMEM prior to addition of [ $^{35}\text{S}$ ]*tran* label (ICN Radiochemicals) to a final concentration of 30 mCi per ml. Cells were pulse labeled for 30 min prior to lysis in RSB/NP-40 buffer (10 mM Tris-Cl, pH 7.4, 10 mM NaCl, 1.5 mM  $\text{MgCl}_2$ , 1% NP-40). Cells were then vortexed briefly and insoluble material was removed by centrifugation. The supernate was added to an equal volume of SDS-PAGE loading buffer (4 $\times$  stock contains 26 mM DTT, 60 mM Tris at pH 6.8, 40% glycerol, 6% SDS) and heated to 95°C for 3 min before resolution on 12% SDS-PAGE. For biochemical assays requiring detection of minute quantities of viral proteins, radiolabel was incorporated from 3.5 to 5 hpi. Cells were then harvested by centrifugation, washed in cold Earle's balanced salt solution, and resuspended on ice in a hypotonic buffer (10 mM Hepes, pH 7.4, 10 mM KCl, 1.5 mM  $\text{MgOAc}$ , 1 mM DTT) prior to lysis in a Dounce cell homogenizer. The protease inhibitor phenylmethylsulfonyl fluoride was added immediately after lysis to a final concentration of up to 0.5 mM to inhibit nonspecific proteolytic activity. The resulting lysate was centrifuged for 15 min at 10,000*g* and the supernate (S10) was collected and stored at -70°C or further processed as described below.

### Purification of eIF4G-specific cleavage activity

One-step separation of 2A<sup>pro</sup> from eIF4G cleavage activity was obtained using a high-resolution G3000SW gel filtration column (TosoHaas). Proteins in the postribosomal supernate fraction or in the ribosomal salt wash were precipitated by addition of saturated ammonium sulfate to 40% and then resolubilized, as previously described (Brown and Ehrenfeld, 1980). The samples were resuspended in buffer H (Wyckoff *et al.*, 1992), and 500  $\mu\text{l}$  was applied to the column, previously equilibrated in the same buffer. 2A<sup>pro</sup> was detected by SDS-PAGE, followed by autoradiography for up to 25 days. eIF4G cleavage activity was measured as described below.

Radiolabeled S10 lysate was fractionated at 1 ml/min on a Sephacryl S300 column (10  $\times$  440 mm) also equil-

ibrated in buffer H, and 0.5-ml fractions were collected. 2A<sup>pro</sup> was detected on autoradiograms of dried SDS-PAGE gels. 2A<sup>pro</sup> or eIF4G cleavage activity (described below) in the column fractions was then assayed for cleavage of synthetic peptide substrates.

The following procedure was used for high-level purification of eIF4G cleavage activity from infected cells. The RSW A-cut fraction was prepared as described above and applied to a Sephacryl S300 column (10  $\times$  440 mm) equilibrated in a buffer containing 20 mM Tris-Cl, pH 8.0, 30 mM KCl, 10% glycerol. Eluted fractions containing peak eIF4G cleavage activity were pooled and subjected to Fast Q Sepharose (Pharmacia) anion exchange chromatography (buffered by 25 mM KCl, 20 mM Tris, pH 8.0, 10% glycerol). Bound proteins were eluted in a gradient of 0.025 to 1 M KCl in the same buffer. Again, fractions containing cleavage activity were pooled and applied to a 1-ml nickel affinity column (Hi-Trap IMAC, Pharmacia) adjusted to 100 mM NaCl, 20 mM Tris-HCl, pH 8.0, 10% glycerol. Bound proteins were eluted with a 25 to 50 mM stepwise gradient of EDTA in the same buffer, and fractions exhibiting eIF4G cleavage activity were combined. Finally, this sample was applied to a 1-ml heparin-Sepharose (HiTrap Heparin, Pharmacia) column, to which bound the majority of total protein, and cleavage activity was detected only in the flow-through fraction.

### Measurement of eIF4G cleavage activity

eIF4G cleavage activity was detected by combining 90  $\mu\text{l}$  of the indicated column fractions with 7  $\mu\text{l}$  of RSW from uninfected HeLa cells, which is highly enriched for eIF4G. One hundred-microliter reactions also contained 5 mM  $\text{MgCl}_2$  and 50 mM NaCl and were incubated 1.5 to 2 h at 37°C. Resulting samples were boiled in SDS-PAGE loading buffer and resolved on 7% SDS-PAGE, then transferred to nitrocellulose and subjected to immunoblot analysis using a polyclonal antiserum raised against human eIF4G. Cleavage assays on samples pooled during high-level purification of eIF4G cleavage activity were incubated for 2 h at 37°C and contained the following: 7  $\mu\text{l}$  eIF4G substrate (dialyzed RSW from uninfected cells), 30  $\mu\text{l}$  of active material pooled from the respective columns, and 5  $\mu\text{l}$  of salt buffer containing 50 mM  $\text{MgCl}_2$  and 0.5 M NaCl. The RSW used in these assays was dialyzed for 2 h at 4°C in buffer containing 20 mM Tris-Cl, pH 7.6, 100 mM KCl, 7 mM 2-ME, 0.2 mM EDTA, and 10% glycerol. Then 150  $\mu\text{l}$  from each column pool was further analyzed on 12% SDS-PAGE and autoradiographed for 4 or 19 days, as indicated in the text and Fig. 2. In certain instances, dilutions of cellular fractions were tested for cleavage activity by immunoblot analysis and percentage cleavage was determined by densitometric analysis of scanned immunoblots with NIH Image software.

## Peptide cleavage assays

Sephacryl S300 column fractions (90 ml) were individually combined with 20 mg of a peptide derived either from the native 2A cleavage site in the viral polyprotein (1D2A) or from the previously mapped 2A<sup>pro</sup> cleavage site on human eIF4G (H-4G). The 150-ml reactions were incubated for 2 h at 30°C. Samples were processed essentially as described previously (Bovee *et al.*, 1998), except reactions were stopped with HClO<sub>4</sub> instead of trichloroacetic acid. Peptides were resolved on a C-18 Ultrasphere reverse-phase HPLC column (Beckman) as described previously (Bovee *et al.*, 1998).

## Quantitation of 2A<sup>pro</sup> from infected cells

Infected HeLa cells were harvested at 5 hpi and lysed in a Dounce cell homogenizer as described above. The lysate was fractionated by centrifugation into soluble (S10) and insoluble (P10) compartments. These fractions were then subjected to SDS-PAGE followed by chemiluminescent Western blotting (Super Signal Ultra, Pierce) in comparison with a dilution series of recombinant PV 2A<sup>pro</sup> previously quantitated by Coomassie stain. X-ray film (type MR, Kodak) was exposed to the luminescent immunoblot for 4 min and developed.

## Poliovirus infection in the presence or absence of 2 mM guanidine

HeLa cells were infected with poliovirus, thoroughly mixed, and then divided equally into two tubes. A 1000-fold dilution of 2 M guanidine-HCl in distilled water was immediately added to one of the tubes, and the split infections were gently stirred in a 37°C recirculating water bath. Two samples were taken from each tube at 20-min intervals from 0 to 5 hpi. One aliquot (5 × 10<sup>5</sup> cells) was pulse labeled for 20 min by resuspension in 100 ml methionine-free medium containing 50 mCi/ml [<sup>35</sup>S]*tran* label and processed as described above, prior to 12% SDS-PAGE and autoradiography. The second aliquot (1 × 10<sup>6</sup> cells) was quickly resuspended in the lysis buffer and processed in the manner described above, in preparation for analysis on 7% SDS-PAGE followed by immunoblotting for eIF4G.

## Immunoprecipitation of 2A<sup>pro</sup>

Fifteen microliters of each 20-ml pulse label sample was diluted into 1.5 ml ice-cold IP buffer (10 mM Tris-Cl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1% NP-40), to which 2.5 ml of polyclonal 2A<sup>pro</sup> antiserum (Benton *et al.*, 1995) was added, and rocked overnight at 4°C. One hundred microliters of a 10% Protein A-agarose (Sigma) slurry, in the same buffer, was added to each sample and rocked for an additional 4 h at 4°C. The Protein A beads were then sedimented and washed five times in cold IP wash buffer (50 mM Tris-Cl, pH

7.4, 500 mM NaCl, 5 mM EDTA, 5% sucrose, 1% NP-40) and then boiled in 20 ml SDS-PAGE loading buffer for 12% SDS-PAGE analysis and autoradiography. X-ray film was exposed for up to 5 weeks to assure detection of 2A<sup>pro</sup> near background levels.

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